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Targeting of Green Fluorescent Protein to Secretory Granules in Oxytocin Magnocellular Neurons and Its Secretion from Neurohypophysial Nerve Terminals in Transgenic Mice

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Oxytocin (OT) is a hypothalamic nonapeptide that is synthesized as part of a larger precursor protein that also contains an approximately 10-kDa protein called neurophysin at its C-terminus. This precursor protein is trafficked through the regulated secretory pathway into secretory granules and then axonally transported to and secreted from nerve terminals in the neural lobe of the pituitary. In this paper, we show that the AI-03 transgene that contains enhanced green fluorescent protein (EGFP) fused to the end of the neurophysin at the C-terminus of the OT pre-prohormone, is expressed selectively in OT-magnocellular neurons and is trafficked to secretory granules in transgenic mice. The EGFP-containing secretory granules are then transported to OT-neurosecretory terminals in the neurohypophysis, where the EGFP fluorescence undergoes depolarization-induced calcium-dependent secretion. The endogenous fluorescence in the neural lobes is sufficiently intense to image secretory events in individual OT nerve terminals (neurosecretosomes) isolated from the posterior pituitaries in these transgenic mice. (*Endocrinology* 143: 1036–1046, 2002)

TRANSGENIC MOUSE MODELS have been extensively used for the study of gene regulation and function in the central nervous system (1). With the discovery of fluorescent reporter proteins such as green fluorescent protein (GFP) (2, 3), it has been possible to employ cell-specific promoters to target these fluorescent reporters to specific neuronal subtypes to identify them in brain slices *in situ* for physiological analysis (4–6). This approach has been particularly successful for the visualization and study of GnRH neurons, in large part, because the cell-specific enhancer elements in the GnRH gene are relatively well understood (7–10). As a result, several laboratories have generated transgenic mice expressing GFP in GnRH neurons and have used these neurons' fluorescence to identify them in brain slices for detailed studies of their synaptic spontaneous electrical activities (5, 11–13).

Targeted gene expression in the oxytocin (OT) and vasopressin (VP) magnocellular neurons (MCNs) of the hypothalamo-neurohypophysial system (HNS) has also been achieved (reviewed in Refs. 14–16). However, the minimal cell-specific enhancer elements in these genes that are responsible for their cell-specific expression are still unclear, and all the successful constructs thus far have contained all the introns and exons of the native genes. Transgenic constructs have been made containing reporters at various exonic sites, and the consensus is that placement of the reporter gene after exon III is the most efficacious site for gene expression (17–19). Consequently, Young *et al.* (19) placed an enhanced GFP (EGFP) reporter gene after exon III in the oxytocin gene, as an in-frame fusion with the carboxyl terminus of the OT pre-prohormone. This construct was similar to an OT-VP minilocus configuration (called V1) that had previously been successful in producing robust expression of a rat transgene (but with no reporter) specifically in OT-MCNs (20). The resulting OT-EGFP transgenic mouse line, designated AI-03, showed cell-specific expression of the EGFP reporter only in OT-MCNs (19).

In the absence of a reporter in exon III, the transgenic mice containing the V1 transgene showed, in addition to its cell-specific expression, very efficient targeting of the rat gene to the secretory granules in the OT-MCNs (21). One study on the intracellular trafficking of a vasopressin pre-prohormone construct that had a full length chloramphenicol acetyl transferase (CAT) reporter attached to its C terminus showed that the reporter protein was targeted to secretory granules *in vivo* (22). This suggested that the similar length EGFP in the OT pre-prohormone of the AI-03 mouse being studied here would exhibit a similar trafficking pattern. Indeed, the initial report that the EGFP in the AI-03 transgenic mouse was detectable in OT containing regions of the posterior pituitary (19) is consistent with this expectation. In this paper, we have examined the specificity and efficiency of the expression of the EGFP protein in OT-MCNs in the AI-03 mouse and have especially focused on its intracellular localization. We find that it is trafficked to secretory granules in OT neurons and transported in these vehicles to OT nerve terminals in the posterior pituitary, where the calcium-dependent secretion

Abbreviations: CAT, Chloramphenicol acetyl transferase; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; HNS, hypothalamo-neurohypophysial system; ir, immunoreactivity; LDCVs, large dense core vesicles; MCNs, magnocellular neurons; NP, neurophysin; OT, oxytocin; VP, vasopressin.

of the EGFP can be demonstrated in neurosecretomes by fluorescence imaging techniques.

Materials and Methods

Transgenic mice

The AI-03 transgenic mouse line which expressed EGFP selectively in hypothalamic oxytocin MCNs (19) was used in these studies. The transgene construct (7573 bp) had an EGFP coding region attached in frame in the middle of exon III, after the entire oxytocin and bulk of the neurophysin coding regions (Fig. 1A). The transgenic mice used in these experiments were primarily heterozygous, and similar data were obtained for the three homozygous mice that were used. All procedures were carried out in accordance with the NIH guidelines on the Care and Use of Animals and animal study protocols approved by the NINDS and NIMH Animal Care and Use Committees.

Immunohistochemistry and fluorescence microscopy

Light microscopic immunohistochemistry was performed on 12-μm fresh frozen sections obtained from adult mouse brains and pituitaries that were rapidly removed and buried in dry ice. They were cut using a 2800 Frig-cut Leica Corp. cryostat and mounted on gelatin coated slides that were stored at −80 C. Before immunostaining, the sections were placed into 4% formaldehyde for 15 min, washed in PBS, and placed in 1% BSA in PBS for 30 min to block nonspecific absorption of the antibodies. The primary antibodies were applied overnight at 4 C. The primary antibodies that were used were two rabbit polyclonal anti-GFP antibodies [CLONTECH Laboratories, Inc., Palo Alto, CA, catalog no. 8367-1 and AB290, (AbCom, Cambridge, UK)], the mouse monoclonal antibodies PS38 and PS41 (23, 24) against OT- and VP-associated neurophysin respectively, at dilutions of 1:10–100, and the rabbit polyclonal antibody, THR (obtained from Dr. Alan Robinson, UCLA School of Medicine, Los Angeles, CA) against VP-associated neurophysin used at a dilution of 1:2000 (25). The secondary antibodies used were goat antirabbit IgG conjugated to Alexa 488 (lot no. 6683-1) for green fluorescence and goat antirabbit IgG conjugated to Alexa 594 (lot no. 6591-1) for red fluorescence (Molecular Probes, Inc., Junction City, OR).

Neurosecretosomes that were attached to poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde in PBS for 1–2 h. After several washes in PBS, the preparations were incubated in PBS containing 0.1% Triton X-100 and 10% normal goat serum overnight. Double immunofluorescence staining was carried out sequentially employing a rabbit polyclonal antibody, THR, or monoclonal antibody PS41 against VP-neurophysin (VP-NP), and the monoclonal antibody, PS38, against OT-neurophysin (OT-NP). The EGFP fluorescence in the neurosecretosomes was sufficiently intense so that immunocytochemical staining for the EGFP was not necessary.

Fluorescence microscopic observations and photographs were made using either a Nikon 5′ eclipse E-400 microscope with a SONY Digital photo camera DKC-5000 system or a Carl Zeiss LM 410 confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

Electron microscopic immunocytochemistry

After dissecting the neural lobe tissue free from the anterior lobe of the pituitary, it was immersion fixed in 1.0% glutaraldehyde/formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at 4 C. The tissue was processed according to the protocol of Phend *et al.* (26) with the following modifications: the filipin and digitonin step was removed, no propylene oxide was used in the dehydration, all steps up to the ethanol were done at 4 C, and the tissue was embedded in Epon. The blocs were polymerized at 60 C for 48 h.

Before immunocytochemistry, a bloc was placed into an unmasking solution of 0.1 M sodium citrate (pH 5.8) solution that was microwaved in a Pelco 3450 oven purchased from Ted Pella Inc. (Redding, CA) at full power for three cycles of 5 min each with a 1-min break between each cycle (27). The bloc and the sodium citrate were allowed to equilibrate to room temperature, at which time the bloc was removed and allowed to air dry. Thin sections were made using a Reichert Ultracut microtome (Leica, Solms, Germany) and placed on single slot nickel grids. All remaining steps were done at room temperature. Sections, on the grids, were etched for 30 min with saturated sodium meta-periodate, then floated on a drop of blocking solution, 0.05 M TBS (pH 7.5) with 0.05% Triton X-100, 1% goat serum, and 1% BSA for 1 h. Double-labeling was performed by mixing the two antibodies, the PS38 Mab against oxytocin-neurophysin diluted 1:15 and the EGFP antibody (CLONTECH Laboratories, Inc.) diluted 1:10 in the blocking solution. The grid was placed onto a small drop of the diluted antibodies in a moist chamber for 3 h. The grid was then passed through three drops of blocking solution for 1 min each and then onto the two colloidal golds mixed together; goat antimouse IgG (10 nm gold) diluted 1:15, and goat antirabbit IgG (20 nm gold) diluted 1:20, in the blocking solution for one hour. Both of the colloidal gold IgGs were purchased from Ted Pella, Inc. Controls were performed by omitting the primary antibodies. Grids were dipped twice into distilled water, air dried, and counterstained with uranyl acetate and lead citrate. The grids were viewed and micrographs recorded in the JEOL 100CX.

Preparation of neurosecretosomes for fluorescence imaging

The neural lobe was dissected out from a single animal, placed in 60 μl of an isotonic sucrose solution (270 mM sucrose; 20 mM HEPES; 100 μM EGTA, pH 7.4 (28, 29), and homogenized in a small glass-homogenizer at room temperature. The suspension (~60 μl) was spread on to two 16-mm diameter glass coverslips precoated with poly-L-lysine. After 15-min incubation, the neurosecretosomes were attached to the coverglass surface and the suspension medium was replaced by control saline (in mM: 140 NaC1; 5 KC1; 2 $CaC1_2$; 1 $MgC1_2$; 10 glucose; 5 HEPES, pH 7.4, which contained 0.15% BSA) and incubated for 2–3 h at 37 C before use in physiological experiments.

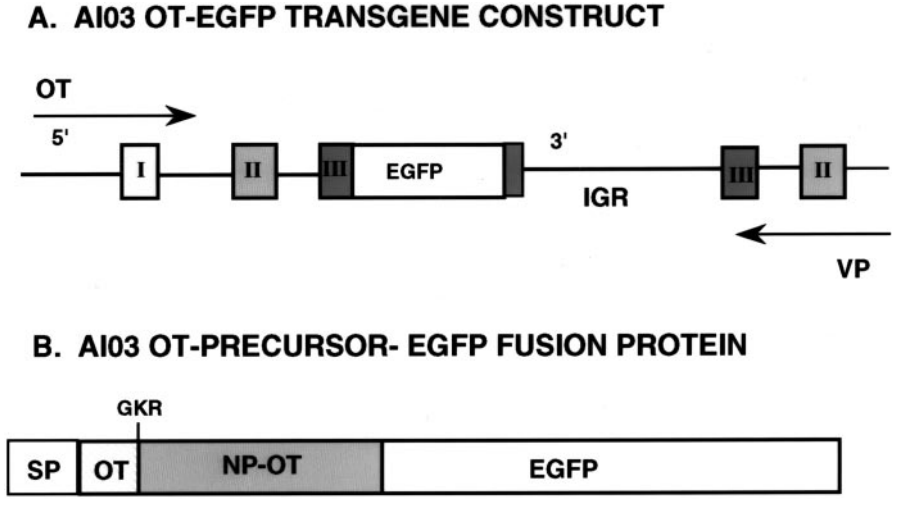

FIG. 1. Schematic views of the AI-03 transgene (A) and the predicted EGFP-tagged OT-precursor protein (B) expressed from this transgene. A, The AI-03 transgene containing three OT-gene exons (I, II, III) separated by the 3.6-kb intragenic region (IGR) from exons II and III of the VP-gene. B, The EGFP-tagged OT-precursor protein expressed from the AI-03 transgene illustrated in A. The positions of the secreted peptide components, OT, NP-OT, and the EGFP adduct are shown. SP, Signal peptide; NP-OT, OT-associated NP (modified from Ref. 19).

Digital fluorescence microscopy and Fura-2 measurements

A glass coverslip containing attached neurosecretosomes was mounted in a bath (700 μl volume) on the stage of upright fluorescence microscope (Carl Zeiss Axioscope, 63× water immersion objective lens) and perfused with control saline at 3 ml/min keeping the bath temperature between 33 and 36 C. Several fluorescent, relatively large secretosomes (>3 μm in diameter) could be found within the illumination field and these were selected for recording EGFP intensity changes. The secretosomes were excited at 488 nm (0.1-sec illumination period at 5-sec cycles) and the emitted light was collected through the 510 nm filter by an intensified charge-coupled device camera (Attofluor Ratiovision, Atto Instruments, Rockville, MD). During recording, the perfusion solution was typically switched from Ca^{2+}-containing normal saline to Ca^{2+}-free medium with secretagogues (cf., 4 ml of 50 and 90 mM K-salines). Each period of the perfusion lasted 100 sec. After recording, data obtained from individual secretosomes was normalized (ΔF/F), where the ΔF is the change in fluorescence intensity during tests and the F is the average fluorescence intensity before the test. To observe changes in intracellular calcium concentration ($[Ca^{2+}]_i$) of the same secretosomes during the recording of EGFP intensity change, the neurosecretosomes were pretreated with 2 μM Fura-2 acetoxymethylester (Molecular Probes, Inc.) for 15 min at 37 C followed by washing for 20 min with Fura-2-free control saline. These secretosomes were excited alternately at three wavelengths (340 nm and 380 nm for Fura-2, and 488 nm for EGFP; with 0.12-sec switching intervals) using 10 nm band-pass interference filters (340HT15, 380HT15, and DF 490HBW; Omega Optics, Brattleboro, VT). Fluorescence emissions were passed through a dichroic mirror, which had two coatings to reflect both EGFP and Fura-2 signals (Chroma 505DCXRU) and a long pass emission filter that had a sharp cut-off below 500 nm. Thus, EGFP and Fura-2 fluorescence intensity changes (average gray level of pixels within the region of interest) were recorded alternately. To reduce illumination intensities of 380 and 488 nm, neutral density filters (OD = 0.2 and 0.5, respectively) were placed in front of these excitation filters. The ratios (340 *vs.* 380 signals) were converted by employing two point calibration method to obtain free $[Ca^{2+}]$ from the equation $[Ca^{2+}]=K_d[R-R_{(Lo)}/R_{(H)}-R] \times Den_{(Lo)}/Den_{(H)}$ in which K_d is the dissociation constant for the dye and taken to be 224 nM (30, 31). The *R* is the measured ratio. The $R_{(H)}$ and $R_{(Lo)}$ are ratio values for high concentration and low concentration standards, respectively. The $Den_{(H)}$ and $Den_{(Lo)}$ are respectively denominator wavelength intensity for high and low concentration standards.

Drugs and solutions

Compositions of the homogenization solution for preparing the neurosecretosomes and the perfusion salines are described above. High K-salines (50 and 90 mM) were prepared by replacing equimillimolar NaC1. Ca^{2+}-free saline was prepared by replacing 2 mM $CaC1_2$ in the control saline with equimolar $MgC1_2$ and adding 0.5 mM EGTA. D-Ala^2-dynorphin A was purchased from Peninsula Laboratories, Inc. (San Carlos, CA). Osmolarity of all salines was kept between 302 and 315 milliosmoles.

Results

Specificity and efficacy of expression of EGFP in AI-03 transgenic mice

Of five transgenic AI03 mouse lines generated, we chose to work with the mouse line that had the most robust and specific expression of the EGFP containing transgene (Fig. 1A), as determined by *in situ* hybridization histochemistry (19). In this study, we have focused on the extent of expression and localization of the fluorescent protein (Fig. 1B) that was expressed in this transgenic mouse line.

Figure 2 shows a typical pattern of expression of the EGFP in the AI-03 mouse hypothalamus. The EGFP protein was found exclusively in magnocellular oxytocin neurons in the PVN (Fig. 2A) and SON (Fig. 2B) of the hypothalamus, confirming the previous report about the absence of ectopic expression of this transgene in the AI03 transgenic line (19). Further examination of the specificity of this expression was made by double-label immunocytochemistry and by comparing the EGFP-immunoreactivity (ir) with that of the immunoreactivity of the specific neurophysins (*i.e.* OT- or VP-associated neurophysin) expressed by the MCNs (Fig. 3). By using highly specific monoclonal antibodies directed against the OT-NP and VP-NP in immunohistochemistry (23, 24), it became apparent that many MCNs in the PVN and SON that expressed the EGFP-ir also expressed OT-NP-ir (Figs. 3, A and B). In contrast, few VP-NP-ir MCNs were found to clearly express EGFP-ir (a rare example is shown at the arrow in Fig. 3C). Overall, there is a selective EGFP expression of the transgene in oxytocin as opposed to vasopressin MCNs, and this is supported by the quantitative analysis of the colocalizations of both peptides and the EGFP-ir shown in Table 1A. Approximately 41% of the 35 OT neurons sampled in an average section contained EGFP-ir, whereas only 2% of the VP neuronal population contained EGFP-ir (Table 1A). The latter finding of a 2% incidence of expression of the OT-transgene (*i.e.* the EGFP-ir) in a VP-MCN is consistent with recent findings that between 1 and 3% of MCNs normally contain comparable levels of both OT and VP mRNA and peptide immunoreactivity (32–34). From these data, one can conclude that the OT-transgene (Fig. 1A) was being expressed selectively in OT-MCNs.

However, while the expression of the AI-03 transgene is selective, it is not very efficient. Only about 41% of the OT neurons in the mouse hypothalamus exhibited detectable EGFP-ir. We presume that this is related to the presence of the reporter in the transgene because a similar rat gene construct that contained only endogenous nucleotide sequences, and no reporter was expressed at a greater than 90% efficiency in transgenic mice (20). In addition to being relatively inefficient, the AI-03 transgene's intensity of expression is very variable. Figure 2 shows that the degree of expression of the transgene in the MCNs (as determined from EGFP-ir) ranged from robust to barely detectable. When the MCNs were examined for intrinsic EGFP fluorescence very few OT-MCNs could be visualized without the aid of immunohistochemistry.

EGFP accumulates in OT nerve terminals in the neural lobe of the pituitary and is present in secretory granules

The expected translated product of the AI-03 transgene contained the entire OT pre-prohormone fused at the C-terminal region of its neurophysins to EGFP (Fig. 1B). If this EGFP would not interfere with the OT-precursor's self-association, which is believed to be involved in its packaging into secretory granules (35, 36), then the green fluorescence should be trafficked to the regulated secretory pathway and transported to nerve terminals in the posterior pituitary. Figure 4 shows cross-sections through the neural lobe, and in contrast to the cell bodies of the MCNs, sufficient EGFP accumulated in the neural lobes so that the intrinsic EGFP fluorescence was relatively robust. The fluorescence in the neural lobe did not require immunohistochemistry to be visualized (Fig. 4A), and was located only in the neural lobe (compare Fig. 4, A and B). Nevertheless, immunohistochem-

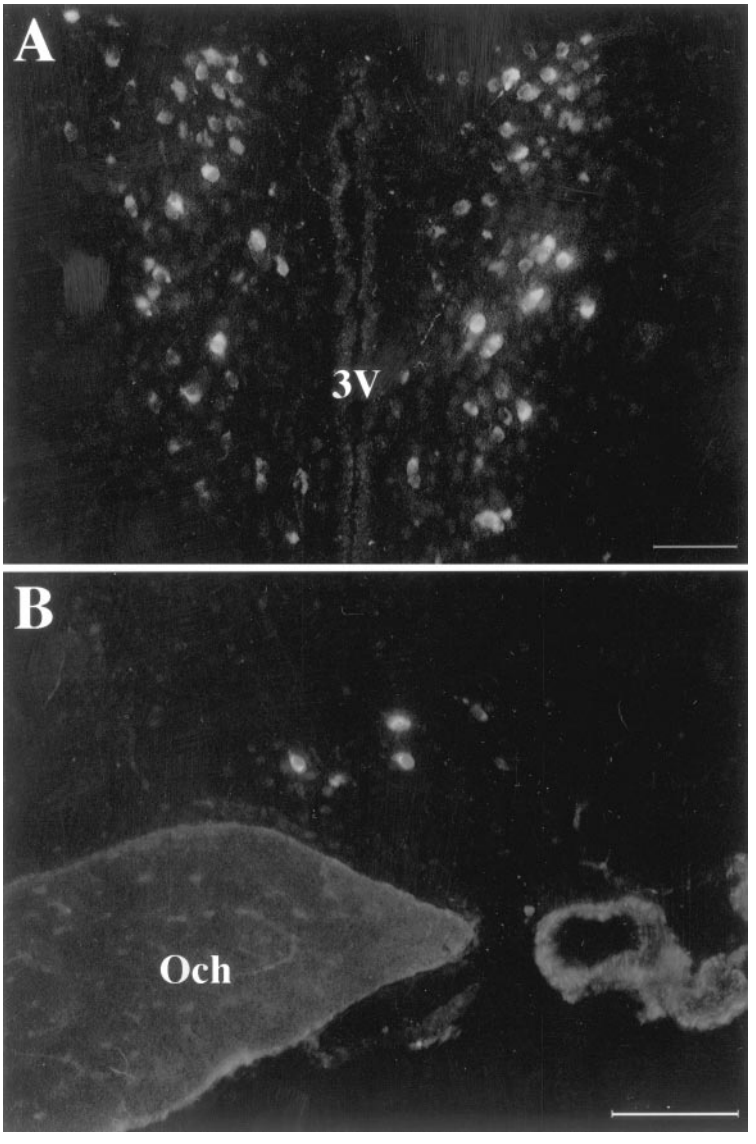

FIG. 2. Expression of EGFP-ir in AI-03 transgenic mouse PVN (A) and SON (B). Note the variability in intensity of the secondary Alexa 488 antibody's fluorescence suggesting variable EGFP gene expression in the MCNs (see *Results*). 3V, Third ventricle; Och, optic chiasms. *Scale lines* in A and B represent 50 μM and 100 μM, respectively.

istry, using an antibody to EGFP and a green fluorescent second antibody did greatly enhance the fluorescent signal observed in the neural lobe (Fig. 4C), and showed the same distribution pattern of fluorescence as observed for the intrinsic EGFP fluorescence. The EGFP was primarily located in the periphery of the neural lobe (Figs. 4, A and C), in a pattern similar to the endogenous OT found in the rat neural lobe (37, 38). When double-label immunocytochemistry was performed using a specific OT-NP antibody (in red fluorescence, Fig. 4C), the merged view illustrated in Fig. 4D shows that the principal EGFP-ir and the predominant OT-NP-ir in the neural lobe clearly overlap. This accumulation of high concentrations of EGFP in the neural lobe in regions corresponding to OT nerve fibers and endings indicates that the EGFP synthesized in the MCN was being transported to the pituitary in a manner similar to the endogenous OT for storage and secretion.

Figure 5 illustrates that when the double-label immunocytochemistry is performed at the electron microscopic level, nerve terminals in the neural lobe contain EGFP (20 nm gold, see *arrows* in Fig. 5A) in the same secretory granules that also contain OT-NP-ir (seen as small, 10 nm gold). Also shown is a non-OT nerve terminal (presumably containing VP) that contains neither label (*arrowhead* in Fig. 5A), and an OT-terminal but with no EGFP (*asterisk* in Fig. 5B). These data indicate that the EGFP is packaged into OT-NP containing secretory granules for axonal transport to the pituitary in these subcellular organelles. Because the secretory granules are also the vehicles for regulated, (calcium-dependent) secretion of OT and OT-NP from nerve terminals in the pituitary, we then examined whether the EGFP fluorescence could be secreted in a calcium-dependent manner.

Secretion of EGFP fluorescence from isolated neurohypophysial nerve terminals

Isolated nerve terminals (neurosecretomes) were generated from AI-03 transgenic mouse neural lobes, and attached onto the surface of coverslips. The sizes of the neurosecretosomes (spherical nerve terminals) derived from the mouse

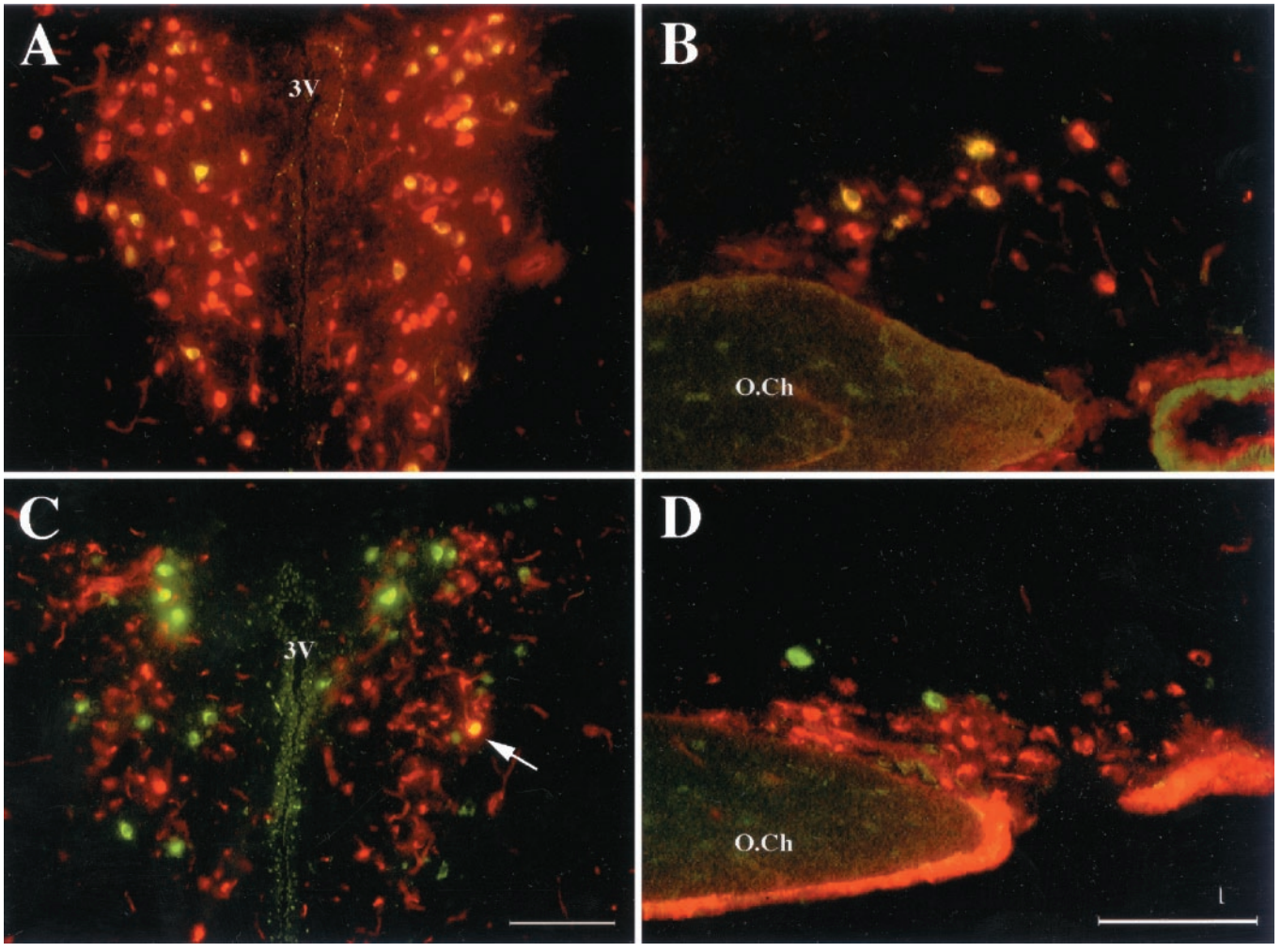

FIG. 3. Merged dual-label immunohistochemical views of EGFP-ir and either OT-NP-ir (A, B) or VP-NP-ir (C, D) in PVN (A, C) and SON (B, D) of AI-03 transgenic mouse. The frozen sections were reacted with the anti-GFP 290 antibody and visualized by an Alexa 488 conjugated secondary antibody in *green* fluorescence. The NP antibodies were visualized as *red* fluorescence, using Alexa 594-conjugated second antibody and colocalization of EGFP and the specific NP is seen as *yellow*. Note that many MCNs show colocalization of NP-OT with EGFP (A, B), whereas only one MCN showed significant colocalization of EGFP and NP-VP (*arrow* in C). *Scale line* in C represents 150 μm in A and D, and *scale line* in D represents 110 μm in B and D.

TABLE 1. Specificity and efficiency of transgene expression in magnocellular neurons of AI 03 transgenic mice

A. Hypothalamic cell bodies	Number of immunoreactive magnocellular neurons/hypothalamic section[a]					
	OT-NP-ir	EGFP-ir+ OT-NP-ir	% OT neurons containing EGFP-ir	VP-NP-ir	EGFP-ir+ VP-NPir	% VP neurons containing EGFP-ir
	35.1 ± 10.6	14.5 ± 4.8	41.3%	47.0 ± 5.9	0.8 ± 0.8	1.7%

[a] N = 12 Sections from 4 Tg mice (at three sections/mouse).

B. Neurosecretosomes (pituitary nerve endings)	Number of immunoreactive neurosecretosomes (NSS) coverslip[b]					
	OT-NP-ir	EGFP+ OT-NP-ir	% OT-NSS containing EGFP	VP-NP-ir	EGFP+ VP-NP-ir	% VP-NSS containing EGFP
	76.6 ± 9.5	22.8 ± 2.9	29.3%	221.2 ± 30.7	1.0 ± 0.2	0.5%

[b] N = 25 coverslips from eight transgenic mouse neurohypophyses.

In A and B, NP-OT-ir and NP-VP-ir were determined using PS38 Mab and PS41 Mab, respectively, and an Alexa 594-conjugated second antimouse antibody (*red color*). EGFP-ir was determined using 290 rabbit anti-EGFP antibody and an Alexa 488 (*green color*)-conjugated second antirabbit antibody in A (see *Materials and Methods*), and intrinsic EGFP fluoresence was recorded in B.

posterior pituitary lobe are variable and are generally smaller than those found in the rat (28, 29), rarely exceeding 4 μm in diameter. The EGFP-containing neurosecretosomes are easily identifiable by their intrinsic fluorescence (Fig. 6).

Double-label immunohistochemistry of transgenic mouse neurosecretosomes comparing their OT-NP-ir (Fig. 6A) and VP-NP-ir (Fig. 6B) with their intrinsic EGFP fluorescence show that colocalization (*yellow*) occurs virtually only in

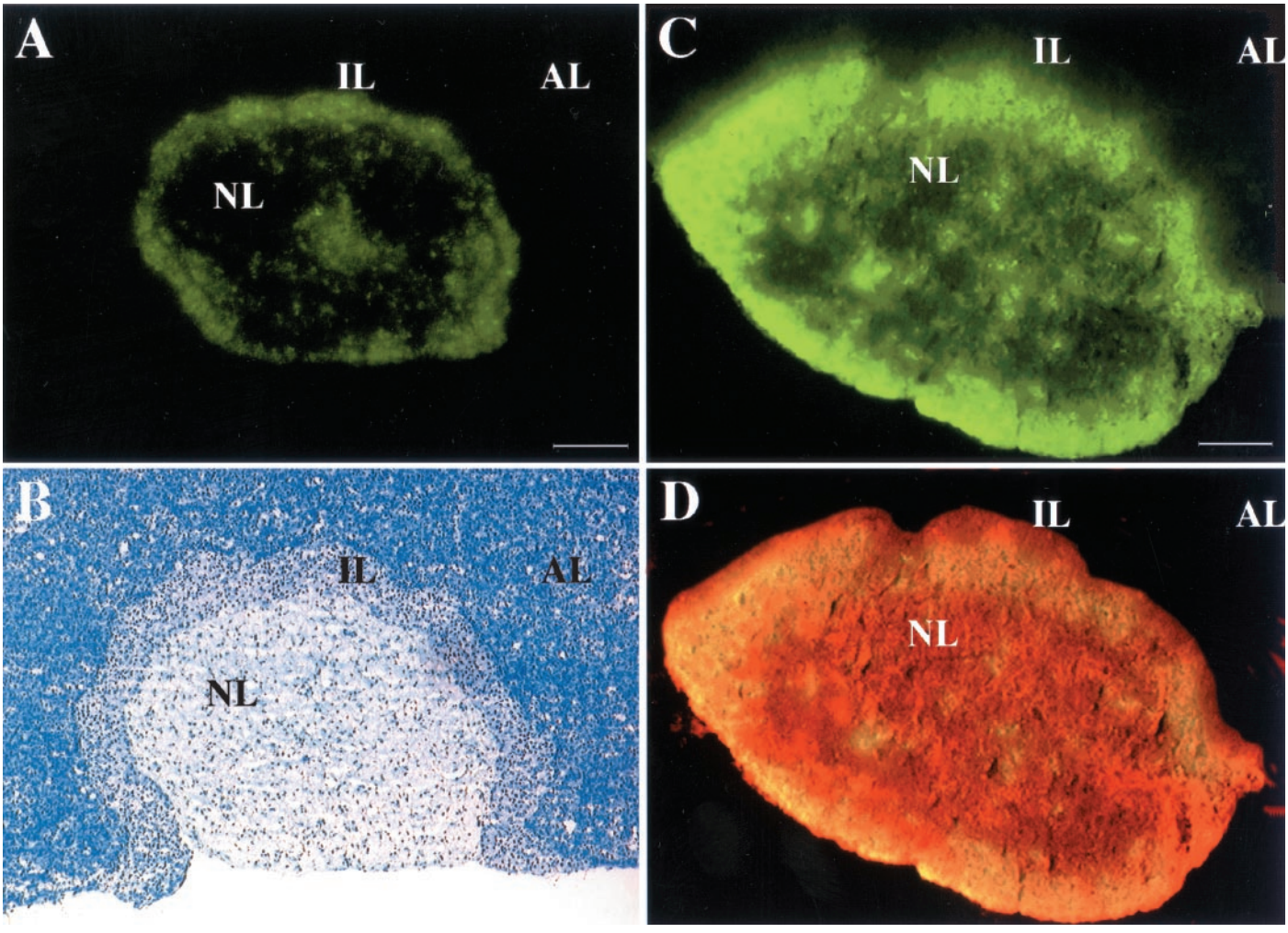

FIG. 4. Endogenous EGFP fluorescence and EGFP-ir is present in the neural lobe (NL) of the mouse pituitary in AI-03 mice. Endogenous EGFP fluorescence in panel A is exclusively found in the NL of the whole pituitary frozen section, shown Nissl stained in panel B. Immunoreactivity of the EGFP in the pituitary section shows increased fluorescence intensity in a pattern in the NL (C), that corresponds to the predominantly peripheral localization of the NP-OT (Refs. 37 and 38). PS38 (NP-OT) immunoreactivity fluorescence found in a merged view of the dual stained NL (D) is consistent with this. NL, Neural lobe; IL, intermediate lobe; AL, anterior lobe. *Scale line* in A represents 60 μm in A and B, and *scale line* in C represents 40 μm in C and D.

OT-NP containing neurosecretosomes (Fig. 6A), and only rarely in the VP-NP containing ones (Fig. 6B). However, as was observed with the cell-bodies of the MCNs (Fig. 3), many OT-NP-containing neurosecretosomes did not contain EGFP. Several smaller sized neurosecretosomes in Fig. 6A appear *green* because in these terminals the OT-NP-ir was present but weak (not shown) and hence, the merged view is biased in favor of the intrinsic EGFP signal. In the quantitative analysis of colocalization in neurosecretosomes that is shown in Table 1B, the *green* and *red* fluorescence signals were recorded independently (to increase the sensitivity of visualization) and compared. These measurements showed that 29% of the OT-NP-ir neurosecretosomes exhibited EGFP fluorescence, whereas only 0.5% of the NP-VP-ir neurosecretosomes contained EGFP fluorescence. These data are consistent with the expression specificities and efficiencies found in the MCNs (Table 1A), indicating a selective but only partial expression of the transgene in the OT-MCNs. The above data were obtained using heterozygous transgenic mice, but similar observations and measurements were made using neurosecretosomes derived from three homozygous (2 females, 1 male) transgenic mice (data not shown).

The intrinsic EGFP fluorescence found in the neurosecretosomes is secreted in response to potassium depolarization in a calcium-dependent manner (Fig. 7). The routine stimulation protocol used (in top abscissa of Fig. 7) involved exposure to high K^+ (50 mM or 90 mM) in normal Ca^{2+}-containing saline, followed by perfusion and stimulation in Ca^{2+}-free saline; and after recovery from Ca^{2+}-free solution, by perfusion with normal Ca^{2+}-containing saline; after which the potassium depolarization was repeated in calcium containing medium. The resulting EGFP intensity changes are shown as the average $\Delta F/F$ and the SEM of five separate measurements. In these experiments, we found that the percentage reduction of the fluorescence intensity ($\Delta F/F$) found in response to application of 90 mM K-saline and 50 mM K-saline were 9.3 ± 1.3% (n = 23), and 5.7 ± 1.3% (n = 18), respectively. In some preparations, intrasecretosomal [Ca^{2+}]

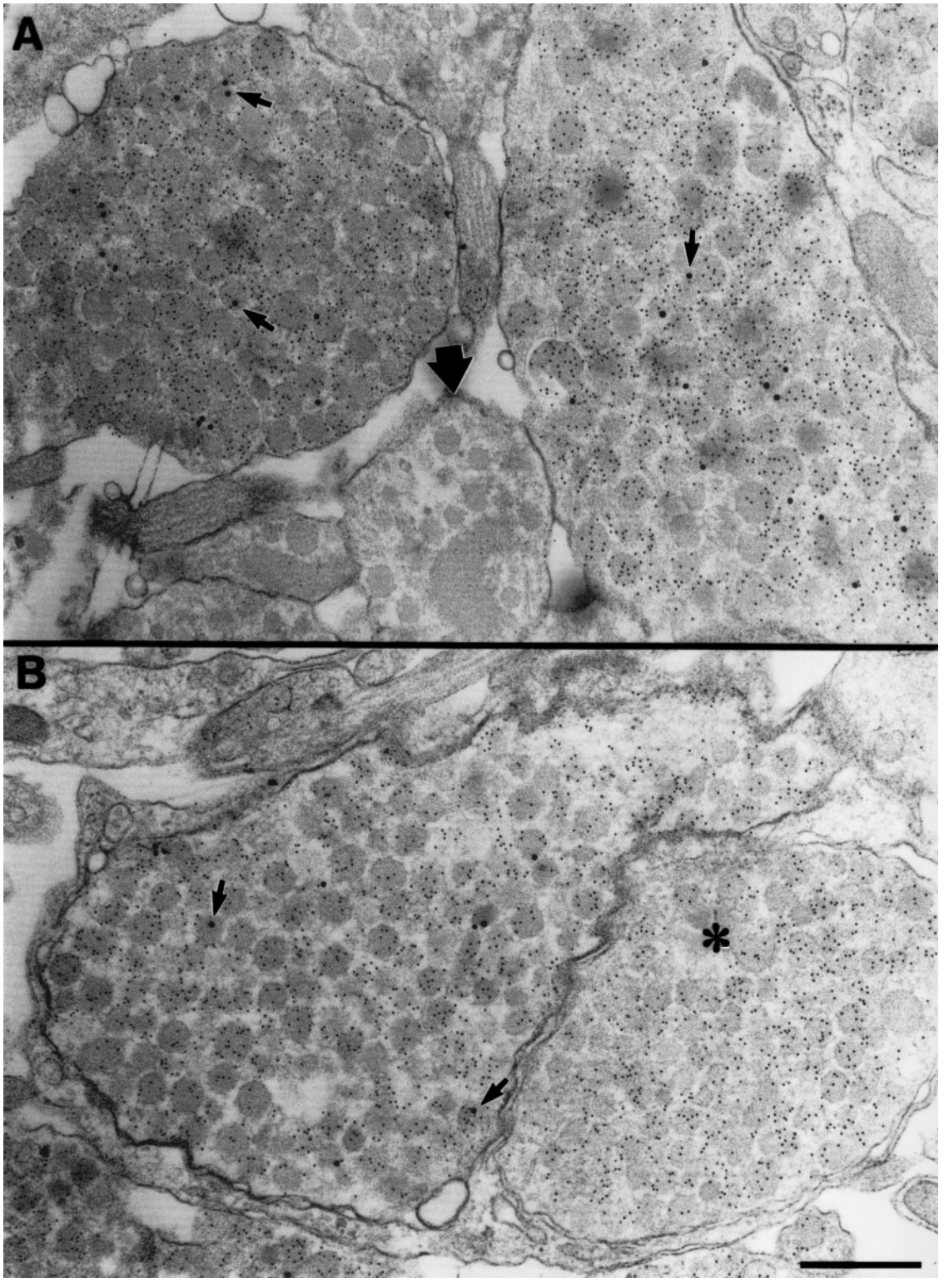

FIG. 5. Double immunolabeling of thin sections of the neural lobe using PS38 (NP-OT) and EGFP antibodies labeled with 10 nm and 20 nm gold particles, respectively. In A a vasopressin terminal (*large arrow*) is negative for both antigens. EGFP-ir (20 nm gold) is indicated by small *arrows*. In B one terminal (*asterisk*) is positive for oxytocin only (*bar*, 500 nm).

changes were also monitored by Fura-2 signals (see *bottom* of Fig. 7). The basal $[Ca^{2+}]$ of secretosomes before stimulation was 63 ± 4.3 nM (n = 19). Application of either 50 or 90 mM K^+-saline for 100 sec in normal $[Ca^{2+}]_o$-saline raised intrasecretosomal $[Ca^{2+}]$ to 828.8 ± 211.0 nM (n = 11). These values are similar to the basal and stimulated $[Ca^{2+}]$ values ob-

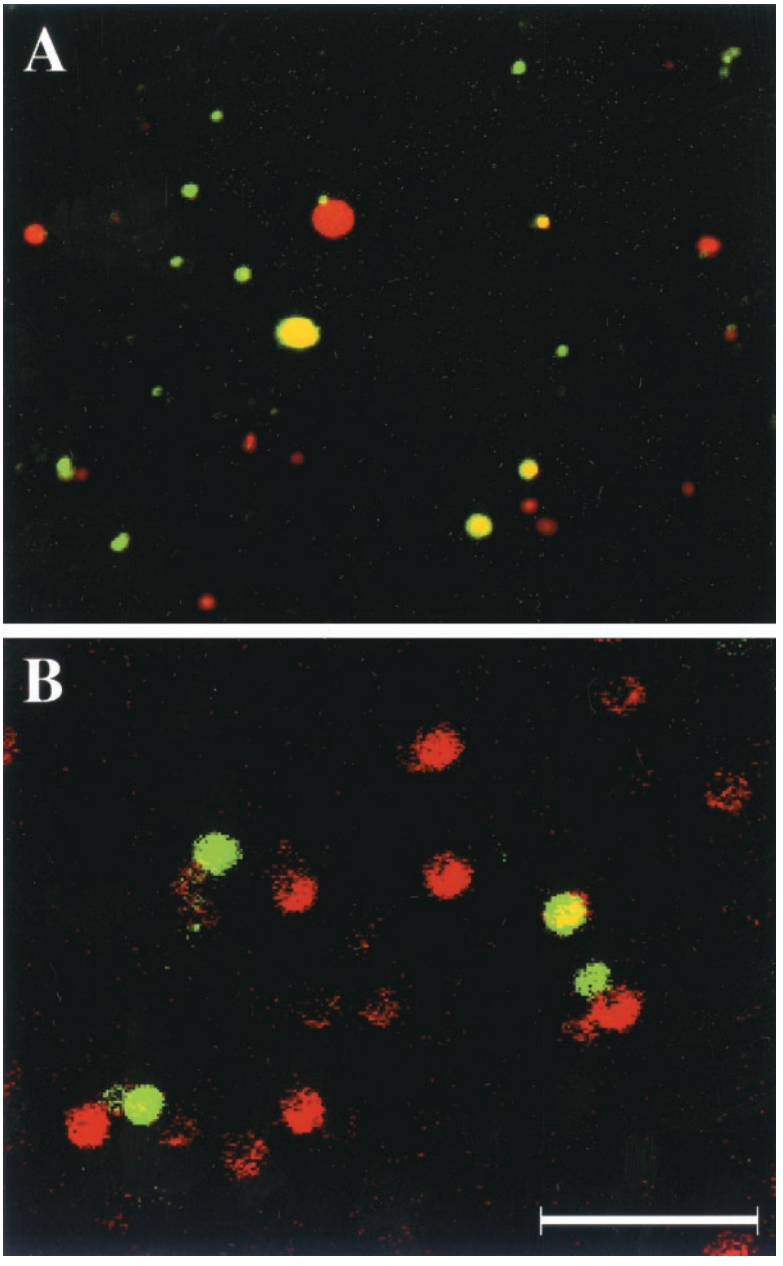

FIG. 6. Neurosecretosomes (nerve terminals) isolated from NLs of AI-03 transgenic mice show intense endogenous EGFP fluorescence (A, B), many of which colocalize with NP-OT-ir (A), but not significantly with NP-VP-ir (B). *Scale line* in B is 20 μM for both A and B.

served in rat neurosecretosomes (39–42). In $[Ca^{2+}]_o$-free saline, neither 50 mM nor 90 mM K^+-saline increased intrasecretosome $[Ca^{2+}]$ or decreased the EGFP fluorescence (Fig. 7).

Discussion

The AI-03 construct, which was integrated into transgenic mice and studied here, contained EGFP as an insert in the third exon of the mouse OT gene that was able to target expression specifically in OT MCNs (19). However, we also found that even in the most robustly expressing transgenic mouse line that contained this transgene, it was only expressed in a minority (~41%) of the OT MCNs (Table 1A). In contrast, the original V1 construct, which did not contain foreign coding sequence as a reporter, was expressed in over 90% of the OT MCNs in transgenic mice (20). Is the reduced expression that was observed with the EGFP constructs due to the influence of the EGFP reporter? In this regard, it is interesting that a similar cell-specific gene expression pattern with a partial penetrance was also observed when a similar OT transgene construct but with a different reporter, CAT was used to produce transgenic mice (22). Therefore, it is unlikely that the low efficiency of transgene expression observed in the OT MCNs in these mice is due to the presence of the particular reporter (CAT or EGFP) used. Furthermore, because this low penetrance was found in multiple founder lines for the EGFP (four lines) and CAT (two lines) constructs, this suggests that integration site factors were not involved.

The principal issue being addressed in this paper is whether the EGFP in the AI-03 transgene would be targeted to the secretory granules in the OT-MCNs, and whether the

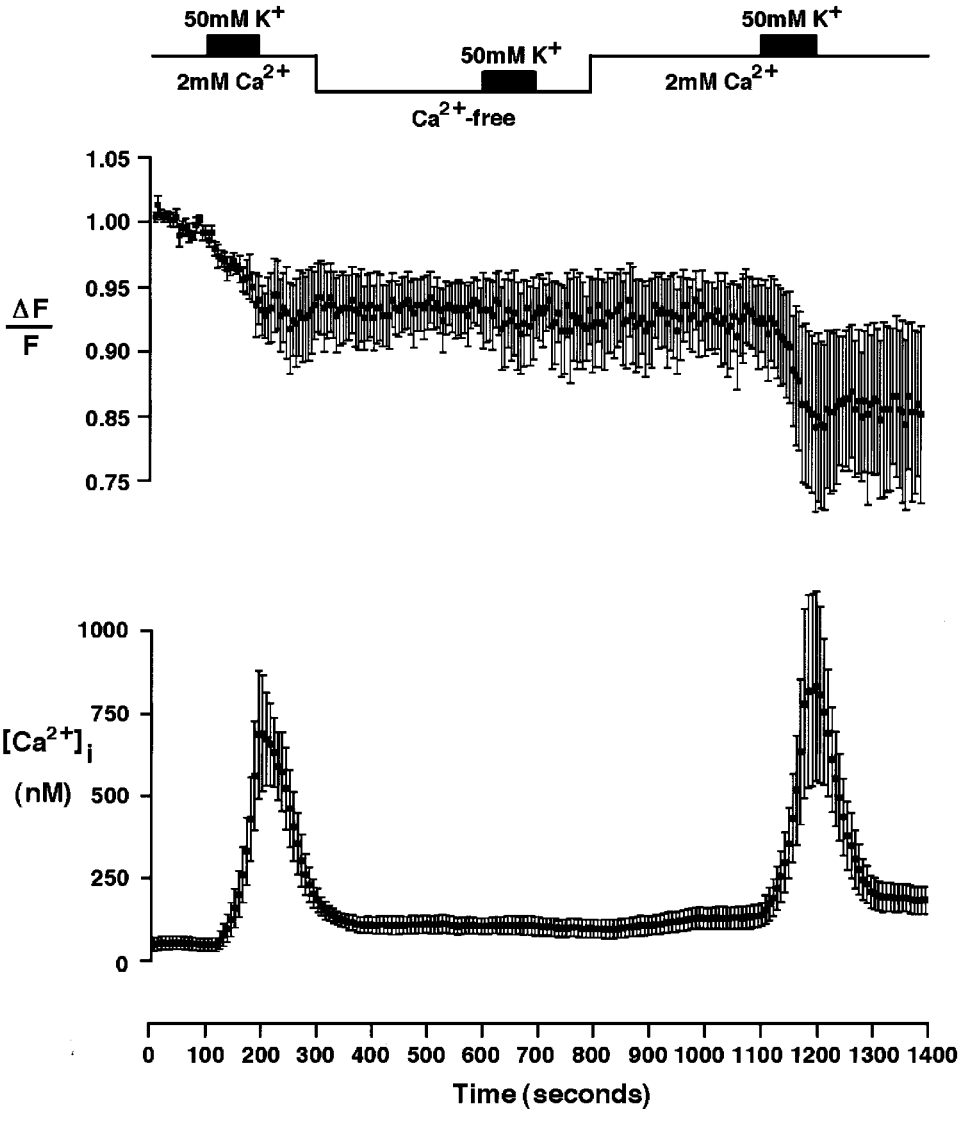

FIG. 7. Depolarization-induced Ca^{2+}-dependent exocytosis in EGFP-containing neurosecretosomes monitored by decreases in EGFP intensity. *Upper record* shows the time course of average intensity decrease ($\Delta F/F$) during applications of 50 mM K^+ in normal $[Ca^{2+}]_o$-containing (2 mM), in $[Ca^{2+}]_o$-free and after washout by normal saline (n = 5, mean ± SEM). The stimulation sequence is shown in the *top abscissa*. *Lower record* shows the corresponding average $[Ca^{2+}]_i$ changes, which were measured alternately with the EGFP intensity changes (n = 5, mean ± SEM). Measurement intervals for EGFP intensity were about 5.6 sec, whereas those for Fura-2 signals were 7.14 sec due to off-line averaging (see *Materials and Methods*).

regulated secretion of the fluorescent proteins in individual neurohypophysial terminals could be visualized by imaging techniques. OT is a nine-amino acid neuropeptide hormone that is synthesized as part of a larger precursor protein (the OT pre-prohormone) in MCNs in the hypothalamus. The OT pre-prohormone contains the N-terminal signal sequence followed by the OT peptide, and an approximately 10-kDa protein called neurophysin at its C terminus. The OT precursor protein is packaged into secretory granules (*i.e.* large dense core vesicles, LDCVs), in which the prohormone is processed and transported to the posterior pituitary for secretion of the peptide products (reviewed in Refs. 15, 43–45). The neurophysin is in a noncovalent association with the neurohypophysial peptides in the secretory granules (35, 46, 47). Based on the biophysical properties of the nonapeptide-neurophysin complex (35) and its crystal structure (48), it was proposed that the self-association properties of this peptide-neurophysin complex as well as the precursor protein might play a critical role in the sorting of the VP precursor to the regulated secretory pathway and the stabilization of the high concentrations of peptides in the LDCVs against disulfide exchange and degradation (35, 49, 50). Several studies supporting the hypothesis that the interaction between the peptide and neurophysin in the protein precursor is necessary for its efficient sorting and packaging to the regulated secretory pathway have been reported (reviewed in Ref. 36). In this report, we tracked the intracellular locations and secretion of the EGFP-tagged, OT-precursor protein to secretory granules in the OT MCNs cells in transgenic mice directly by electron microscopic immunocytochemistry and studied its secretion from individual living cell processes by digital fluorescence imaging techniques.

Very few studies have examined the ultrastructural localization and trafficking of OT- or VP-transgene peptide products. The first was the rat "minilocus" transgene that expressed the wild-type rat OT gene in transgenic mice (20). The rat OT-NP could be discriminated from mouse OT-NP immunocytochemically by using a monoclonal antibody, PS67, which was specific for the rat OT-NP. Both the transgenic rat and endogenous mouse OT-NPs were found to be colocalized in secretory granules in cell bodies and nerve terminals in only the OT MCNs (21). In another study, a rat

VP transgene containing a short epitope tag at the end of exon 3 of the VP gene (18) was found to be trafficked to secretory granules in the posterior pituitaries of transgenic rats (17). In the studies by Jeong *et al.* (22), a full-length CAT reporter was fused to the C termini of either a VP-pre-pro-hormone or OT pre-pro-hormone transgene, and in both cases the reporter was selectively trafficked to secretory granules in the MCNs. In the AI-03 construct being studied here the EGFP moiety would be attached to the end of the OT-NP in the OT prohormone (see Fig. 1B). Because the peptide (OT or VP) and neurophysin interaction in the prohormone is believed to be involved in the sorting process to the regulated secretory pathway (35, 36, 51), it is interesting that the long EGFP adduct attached to the OT-NP did not prevent trafficking of the OT-prohormone-EGFP fusion protein to the secretory granules (Fig. 5). In fact, it is remarkable that the OT prohormone EGFP fusion protein used in this study was effectively sorted to the secretory granules in the MCNs, even though the EGFP reporter was more than two times larger in molecular weight than the entire OT prohormone to which it was fused.

Because in this OT-EGFP transgenic mouse the fluorescent reporter is targeted to secretory granules in the OT magnocellular neurons that are subsequently transported to nerve endings in the neural lobe (Figs. 4 and 5), this provided a unique opportunity to visualize neurosecretion from individual OT nerve terminals. Previous measurements of OT and VP peptide secretion in the HNS, from nerve terminals in the neural lobe (28, 29, 39, 40, 52, 53) or from dendrites in the hypothalamus (54), have been made by RIA of samples, collected either by superfusion *in vitro* from neural lobe or by microdialysis *in vivo* in hypothalamus. While the RIA method is extremely sensitive and has provided much valuable information about OT and VP secretion, the topographic (dendrite *vs.* nerve terminal) and kinetic aspects of the secretion of these peptides cannot be studied by these procedures. In the present study, we have been able to use the AI-03 transgenic mouse and imaging methods by which the secretion process can be directly visualized (55, 56) to demonstrate calcium-dependent secretion from individual OT nerve endings (neurosecretosomes) isolated from their neural lobes (Fig. 7).

Using this paradigm, it should also be relatively easy to perform various pharmacological studies of neurosecretion from individual identified peptidergic nerve terminals that have previously only been done using whole neural lobes or mixed neurosecretosomes and RIA assays (28, 29, 40, 41). For example, κ opioid receptor agonists, such as dynorphin, are known to inhibit depolarization-evoked secretions of OT from rat neural lobes and neurosecretosomes (42, 57–60). In preliminary experiments, we have confirmed that the treatment of neurosecretosomes (derived from AI-03 transgenic mice) with 2.5×10^{-6} M dynorphin A inhibited the 50 mM K^+-induced secretion of EGFP (*i.e.* the fluorescence intensity decrease) in normal Ca^{2+}-containing saline from individual OT nerve terminals (n = 3). This dynorphin effect was reversible after washing out the dynorphin and replacing it with normal saline (data not shown). Given the availability of AI-03 transgenic mice that contain fluorescent secretory granules, and the various dissociated and organotypic tissue cultured neuron models that can be derived from them (25, 61), it should then be possible, in the future, to compare the pharmacologies of secretion from the dendrites and nerve terminals of intact OT-MCNs *in vitro*.

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References

1. **Herrup K** 1995 Transgenic and ES cell chimeric mice as tools for the study of the nervous system. Discuss Neurosci 10:41–64
2. **Chalfie M** 1995 Green fluorescent protein. Photochem Photobiol 62:651–656
3. **Tsien RY** 1998 The green fluorescent protein. Annu Rev Biochem 67:509–544
4. **Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR** 2000 Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41–51
5. **Spergel DJ, Krüth U, Shimshek DR, Sprengel R, Seeburg PH** 2001 Using reporter genes to label selected neuronal populations in transgenic mice for gene promoter, anatomical, and physiological studies. Prog Neurobiol 63:673–686
6. **van den Pol, Anthony N, Ghosh Prabhat K** 1998 Selective neuronal expression of green fluorescent protein with cytomegalovirus promoter reveals entire neuronal arbor in transgenic mice. J Neurosci 18:10640–10651
7. **Mason AJ, Pitts SL, Nikolics K, Szonyi E, Wilcox JN, Seeburg PH, Stewart TA** 1986 The hypogonadal mouse: reproductive functions restored by gene therapy. Science 234:1372–1378
8. **Windle JJ, Weiner RI, Mellon PL** 1990 Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol Endocrinol 4:597–603
9. **Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI** 1990 Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5:1–10
10. **Wolfe AM, Wray S, Westphal, H, Radovick S** 1996 Cell-specific expression of the human gonadotropin-releasing hormone gene in transgenic animals. J Biol Chem 271:20018–20023
11. **Spergel DJ, Krüth U, Hanley DF, Sprengel R, Seeburg PH** 1999 GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. J Neurosci 19:2037–2050
12. **Suter KJ, Song WJ, Sampson TL, Wuarin J-P, Saunders JT, Dudek FE, Moenter SM** 2000 Genetic targeting of green fluorescent protein to gonadotropin-releasing hormone neurons: characterization of whole-cell electrophysiological properties and morphology. Endocrinology 141:412–419
13. **Suter KJ, Wuarin J-P, Smith BN, Dudek FE, Moenter SM** 2000 Whole-cell recordings from preoptic/hypothalamic slices reveal burst firing in gonadotropin-releasing hormone neurons identified with green fluorescent protein in transgenic mice. Endocrinology 141:3731–3736
14. **Waller SJ, Ratty A, Burbach JPH, Murphy D** 1998 Transgenic and transcriptional studies on neurosecretory cell gene expression. Cell Mol Neurobiol 18:149–172
15. **Burbach JPH, Luckman SM, Murphy D, Gainer H** 2001 Gene regulation in the magnocellular hypothalamo-neurohypophysial system. Physiol Rev 81: 1197–1267
16. **Gainer H, Young III WS** 2001 Transgenic models for studies of oxytocin and vasopressin. In: Castro MG, ed. Transgenic models in endocrinology. Chapter 2. Boston: Kluwer-Academic Press; 25–46
17. **Waller S, Fairhall KM, Xu J, Robinson ICAF, Murphy D** 1996 Neurohypophyseal and fluid homeostasis in transgenic rats expressing a tagged rat vasopressin prepropeptide in hypothalamic neurons. Endocrinology 137: 5068–5077
18. **Zeng Q, Carter DA, Murphy D** 1994 Cell specific expression of a vasopressin transgene in rats. J Neuroendocrinol 6:469–477
19. **Young III WS, Iacangelo A, Luo X-ZJ, King C, Duncan K, Ginns EI** 1999 Transgenic expression of green fluorescent protein in mouse oxytocin neurons. J Neuroendocrinol 11:935–939
20. **Young III WS, Reynolds K, Shepard EA, Gainer H, Castel M** 1990 Cell-specific expression of the rat oxytocin gene in transgenic mice. J Neuroendocrinol 2:917–925
21. **Belenky M, Castel M, Young III WS, Gainer H, Cohen S** 1992 Ultrastructural immunolocalization of rat oxytocin-neurophysin in transgenic mice expressing the rat oxytocin gene. Brain Res 583:279–286
22. **Jeong S-W, Castel M, Zhang B-J, Fields RL, Paras P, Arnheiter H, Chin H,**

Gainer H 2001 Cell-specific expression and subcellular localization of CAT-reporter protein expressed from oxytocin and vasopressin gene promoter-driven constructs in transgenic mice. Exp Neurol 171:255–271
23. **Ben-Barak Y, Russell JT, Whitnall MR, Ozato K, Gainer H** 1985 Neurophysin in the hypothalamo-neurohypophysial system. I. Production and characterization of monoclonal antibodies. J Neurosci 5:81–97
24. **Whitnall MH, Key S, Ben-Barak Y, Ozato K, Gainer H** 1985 Neurophysin in the hypothalamo-neurohypophysial system. II. Immunocytochemical studies of the ontogeny of oxytocinergic and vasopressinergic neurons. J Neurosci 5:98–109
25. **House SB, Thomas A, Kusano K, Gainer H** 1998 Stationary organotypic cultures of oxytocin and vasopressin magnocellular neurons from rat and hypothalamus. J Neuroendocrinol 10:849–861
26. **Phend KD, Rustioni A, Weinberg RJ** 1995 An osmium-free method of epon embedment that preserves both ultrastructure and antigenicity for post-embedding immunocytochemistry. J Histochem Cytochem 43:283–292
27. **Inman SA, Young L, Chaiwun B, Taylor CR** 1995 Comparison of two microwave based antigen-retrieval solutions in unmasking epitopes in formalin-fixed tissue for immunostaining. Anticancer Res 15:1153–1158
28. **Cazalis M, Dayanithi G, Nordman JJ** 1987 Hormone release from isolated nerve endings of the rat neurohypophysis. J Physiol 390:55–70
29. **Cazalis M, Dayanithi G, Nordmann JJ** 1987 Requirements for hormone release from permeabilized nerve endings isolated from the rat neurohypophysis. J Physiol 390:71–91
30. **Grynkiewicz Z, Poenie M, Tsien RY** 1985 A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. Biol Chem 260:3440–3450
31. **Williams DA, Fay FS** 1990 Intracellular calibration of the fluorescent calcium indicator Fura-2. Cell Calcium 11:75–83
32. **Mezey E, Kiss JZ** 1991 Coexpression of vasopressin and oxytocin in hypothalamic supraoptic neurons of lactating rats. Endocrinology 129:1814–1820
33. **Glasgow E, Kusano K, Chin H, Mezey E, Young III WS, Gainer H** 1999 Single cell reverse transcription-polymerase chain reaction analysis of rat supraoptic magnocellular neurons: Neuropeptide phenotypes and high voltage-gated calcium channel subtypes. Endocrinology 140:5391–5401
34. **Xi D, Kusano K, Gainer H** 1999 Quantitative analysis of oxytocin and vasopressin mRNAs in single magnocellular neurons isolated from supraoptic nucleus of rat hypothalamus. Endocrinology 140:4677–4682
35. **Breslow E, Burman S** 1990 Molecular, thermodynamic, and biological aspects of recognition and function in neurophysin-hormone systems: a model system for the analysis of protein-peptide interactions. Adv Enzymol 63:1–67
36. **de Bree FM** 2000 Trafficking of the vasopressin and oxytocin prohormone through the regulated secretory pathway. J Neuroendocrinol 12:589–594
37. **Van Leeuwen FW, Swaab DF** 1977 Specific immunoelectron microscopic localization of vasopressin and oxytocin in the neurohypophysis of the rat. Cell Tissue Res 177:493–501
38. **Van Leeuwen FW, de Raay C, Swaab DF, Fisser B** 1979 The localization of oxytocin, vasopressin, somatostatin and luteinizing hormone releasing hormone in the rat neurohypophysis. Cell Tissue Res 202:189–201
39. **Stuenkel EL** 1990 Effects of membrane depolarization on intracellular calcium in single nerve terminals. Brain Res 529:96–101
40. **Stuenkel EL, Nordmann J** 1993 Intracellular calcium and vasopressin release of rat isolated neurohypophysial nerve endings. J Physiol 468:335–355
41. **Fatatis A, Holtzclaw L, Payza K, Russell JT** 1992 Secretion from rat neurohypophysial nerve terminals (neurosecretosomes) rapidly inactivates despite continued elevation of intracellular Ca^{2+}. Brain Res 574:33–41
42. **Kato M, Chapman C, Bicknell RJ** 1992 Activation of κ-opioid receptors inhibits depolarization-evoked exocytosis but not the rise in intracellular Ca^{2+} in secretory nerve terminals of the neurohypophysis. Brain Res 574:138–146
43. **Brownstein MJ, Russell, JT, Gainer H** 1980 Synthesis, transport, and release of posterior pituitary hormones. Science 207:373–378
44. **Gainer H, Wray S** 1994 Cellular and molecular biology of oxytocin and vasopressin. In: Knobil E, Neill JD, eds. The physiology of reproduction. New York: Raven Press Ltd.; 1099–1129
45. **Sladek CD** 1999 Antidiuretic hormone: synthesis and release. In: John CS Fray, ed. Handbook of physiology, section 7. The endocrine system, vol III. Oxford, UK: Oxford University Press; 436–495
46. **Acher RA, Light A, DuVigneaud V** 1958 Purification of oxytocin and vasopressin by way of a protein complex. J Biol Chem 233:116–120
47. **Pickering BT, Jones CW** 1978 The neurophysins. Horm Prot Pept 5:103–158
48. **Rose JP, Wu CK, Hsiao CD, Breslow E, Wang BC** 1996 Crystal structure of the neurophysin-oxytocin complex. Nat Struct Biol 3:163–169
49. **Chaiken IM, Tamaoki H, Brownstein, MJ, Gainer H** 1983 Onset of neurophysin self-association upon neurophysin/neuropeptide hormone precursor biosynthesis. FEBS Lett 164:361–365
50. **Kanmera T, Chaiken IM** 1985 Molecular properties of the oxytocin/bovine neurophysin biosynthetic precursor. J Biol Chem 260:8474–8482
51. **de Bree FM, Knight D, Howell L, Murphy D** 2000 Sorting of the vasopressin prohormone into the regulated secretory pathway. FEBS Lett 475:175–180
52. **Wang G, Dayanithi G, Kim S, Horn D, Nadaschi L, Kristipati R, Ramachandran J, Stuenkel EL, Nordmann JJ, Newcomb R, Lemos JR** 1997 Role of Q-type Ca^{2+} channels in vasopressin secretion from neurohypophysial terminals of the rat. J Physiol 502:351–363
53. **Sabatier N, Richard P, Dayanithi G** 1997 L-, N- and T- but neither P- nor Q-type Ca^{2+} channels control vasopressin-induced Ca^{2+} influx in magnocellular vasopressin neurones isolated from rat supraoptic nucleus. J Physiol 503:253–268
54. **Neumann I, Russell JA, Landgraf R** 1993 Oxytocin and vasopressin release within the supraoptic and paraventricular nuclei of pregnant, parturient, and lactating rats: a microdialysis study. Neuroscience 53:65–75
55. **Burke NV, Han W, Li D, Takimoto K, Watkins SC, Levitan ES** 1997 Neuronal peptide release is limited by secretory granule mobility. Neuron 19:1095–1102
56. **Kaether C, Gerdes H-H** 1995 Visualization of protein transport along the secretory pathway using green fluorescent protein. FEBS Lett 369:267–271
57. **Bicknell RJ, Leng G** 1982 Endogenous opiates regulate oxytocin but not vasopressin secretion from the neurohypophysis. Nature 298:161–162
58. **Bondy CA, Gainer H, Russell JT** 1988 Dynorphin A inhibits and naloxone increases the electrically stimulated release of oxytocin but not vasopressin from the terminals of the neural lobe. Endocrinology 122:1321–1327
59. **Falke N** 1988 Dynorphin (1–8) inhibits stimulated release of oxytocin but not vasopressin from isolated neurosecretory endings of the rat neurohypophysis. Neuropeptides 11:163–167
60. **Zhao B-G, Chapman C, Bicknell RJ** 1998 Functional opioid receptors on oxytocin and vasopressin nerve terminals isolated from the rat neurohypophysis. Brain Res 462:62–66
61. **Kusano K, House SB, Gainer H** 1999 Effects of osmotic pressure and brain-derived neurotrophic factor on the survival of postnatal hypothalamic oxytocinergic and vasopressinergic neurons in dissociated culture. J Neuroendocrinol 11:145–152